Invited Paper

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A Native-Chemical-Ligation-Based Turn-on Fluorescent Probe for Selective Detection of Cysteine

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Selective and quantitative detection of biological thiols such as cysteine, homocysteine, and glutathione is often necessary because abnormal levels of such thiols can cause some diseases. Here, we report that bis(pentafluorophenyl) 1,4-benzenedicarbothionic acid diester can serve as a turn-on fluorescent probe for selective detection of cysteine vis-a-vis homocysteine and glutathione. When cysteine was added to a mixture of the diester and a sodium phosphate buffer solution with THF (60 vol%), which is non-fluorescent, the mixture became green-fluorescent. In contrast, addition of homocysteine or glutathione did not make the mixture fluorescent. A native-chemical-ligation-based mechanism is proposed.

Keywords: Fluorescence; Sensing; Thiols.

Masaki Shimizu *was born in 1965 in Tokyo, Japan. He received his Ph.D. from the Tokyo Institute of Technology in 1994 under the supervision of Profs. Takeshi Nakai and Koichi Mikami. After working at Mitsubishi Chemical Co. Ltd. for 14 months, he joined Prof. Tamejiro Hiyama's group at the Research Laboratory of Resources Utilization, Tokyo Inst. of Tech. as an Assistant Professor in 1995. In 1998, he moved to Kyoto University as an Assistant Professor. He spent one year (1999~2000) at Massachusetts Institute of Technology as a postdoctoral fellow in the group of Prof. Stephen L. Buchwald and was promoted to Associate Professor of Kyoto Univ. in 2003. In 2012, he became Professor at Kyoto Institute of Technology. His research interest focuses on design and invention of functional π-conjugated molecules as well as the development of synthetic methodologies directed toward functional organic materials.*

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INTRODUCTION

Biological thiols such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) are necessary for various biological processes, and a surplus or deficiency of thiols in the human body can cause some diseases. Hence, accurate monitoring of biothiol levels is useful, and a variety of colorimetric and fluorescent probes for detection of biothiols have been developed to date.[1] The sensing mechanism of such probes is generally triggered by a nucleophilic attack on an electrophilic functional group under strong basic conditions or by coordination with a metal of sulfhydryl groups. Such approaches make it difficult to discriminate Cys and Hcy because their molecular structures are similar, as is their chemical reactivity. Therefore, highly selective detection of either Cys or Hcy is one of the challenging problems in the field of biothiol sensing.[2]

Native chemical ligation (NCL) is widely used as a versatile method for protein backbone synthesis and involves transthioesterification of a peptide α-thioester with a peptide containing an *N*-terminal cysteine, followed by facile rearrangement of the acyl group of the resulting thiocarbonyl moiety from a sulfur to a nitrogen of the cysteine moiety.[3] Because of the mild conditions, high chemoselectivity, and biocompatibility, NCL represents an attractive mechanism for sensing of bioactive materials. On the other hand, real examples of NCL-based probes are scarce.[4] Hence, it would be worthwhile to develop NCL-based probes for selective detection of biological thiols.

We previously developed dimethyl 2,5-bis(diorganoamino)terephthalates (**1**) as novel fluorophores with green-to-yellow emission in solution and in the solid state with a moderate or high quantum yield, respectively (Figure 1).[5] The excited state was confirmed to be caused by the intramolecular charge transfer (ICT) from the diorganoamino group to methoxycarbonyl group, and the luminescent properties depend on the degree of ICT, in other words, on the combination of the donor and acceptor.[6] Accordingly, luminescent properties of thioesters **2** and amides **3** are expected to be largely different because they possess the same electronic structure as that of compound **1** (Figure 1). Because thioesters and the corresponding amides can serve as a starting material and a product of NCL, as stated above, we hypothesized that sensing of biological thiols by means of thioesters **2** as a probe is possible via monitoring of luminescent properties of this system. We were able to prove the hypothesis: here, we report selective sensing of Cys by 2,5-bis(diphenylamino)-1,4-benzenedicarbothionic acid bis(pentafluorophenyl)ester (Figure 1, **2a**).

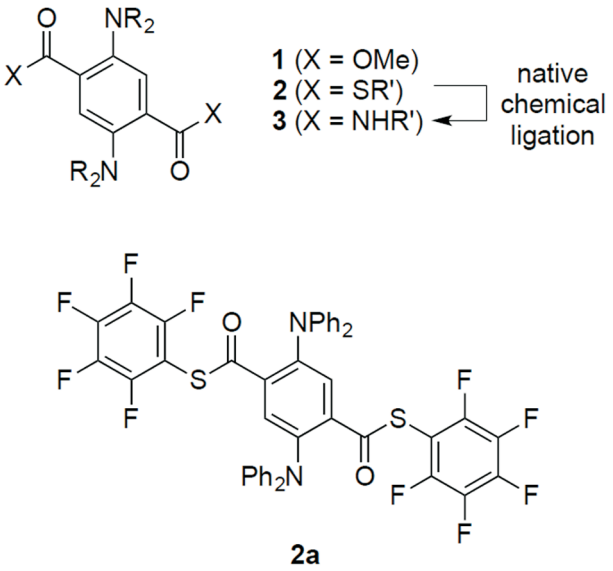
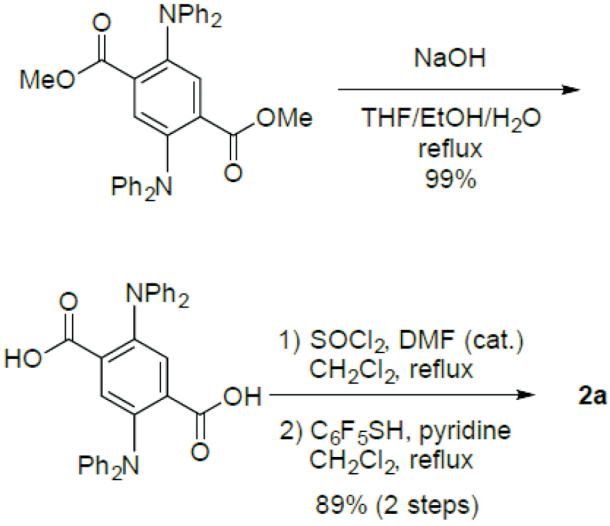


Fig. 1. Molecular structures of 1,4-bis(carbonyl)-2,5-diaminobenzenes **1**–**3**.

RESULTS AND DISCUSSION

Dithioester **2a** was prepared in a high yield from dimethyl 2,5-bis(diphenylamino)terephthalate (**1**: R = Ph) via three steps involving hydrolysis, conversion to an acid chloride, and esterification with C_6F_5SH in the presence of pyridine as a base (Scheme 1). Purification of the crude product by silica gel column chromatography gave **2a** as a red solid with good thermal stability (decomposition point: 234 °C).

Scheme 1 Preparation of dithioester **2a**.



In solution, **2a** was non-fluorescent. In contrast, a powder of **2a** showed red fluorescence with an emission maximum at 642 nm and a quantum yield of 0.21. Thus, **2a** exhibited aggregation-induced emission (AIE).[7] Then, we tested the AIE behavior of **2a** in a mixture of THF and sodium phosphate buffer (PB; 10 mM, pH 7.2). If PB constituted up to 40 vol%, the mixture remained non-fluorescent (Figure 2). When the aqueous fraction constituted more than 40 vol%, the mixture became red-fluorescent with the emission maximum near 615–620 nm, indicating that **2a** started to form aggregates (Figure S1 in Supporting Information). The fluorescence intensity was enhanced as the buffer's proportion increased and reached a maximum at 80 vol% PB. According to these results, further experiments of thiol sensing using **2a** were performed with a mixture of THF (60 vol%) and PB (40 vol%).

When an excess amount of Cys was added to a solution of **2a** in a mixture of THF and PB (60/40), which was non-fluorescent, the resulting solution showed green fluorescence with the emission maximum at 521 nm upon excitation at 390 nm (Figure 3). The fluorescence intensity increased with time up to 10 h, and significant enhancement was confirmed at 2 h after the addition of Cys. Thus, the reaction time was set to 2 h in further experiments.

To evaluate the sensitivity of **2a** to Cys, fluorescence spectra of a mixture of **2a** and Cys in THF/PB (60/40) were recorded at various concentrations of Cys. As shown in Figure 4, the fluorescent signal was enhanced as the Cys concentration increased (see Figure S2 in Supporting Information), and the fluorescence intensity at 521 nm was linearly proportional to the Cys concentration in the range of 0–1 mM ($R^2 = 0.9824$). The limit of detection of Cys was found to be 58 μM on the basis of the signal-to-noise ratio (S/N = 3). The concentration of Cys in serum or plasma of healthy people is typically ~250 μM.[8] Thus, the sensitivity of **2a** is suitable for detection of a Cys deficiency although further improvements are necessary to achieve faster sensing and better sensitivity.

To examine the sensing selectivity of **2a**, Hcy and GSH were subjected to the same conditions as those for Cys sensing. As shown in Figure 5, no fluorescence of the

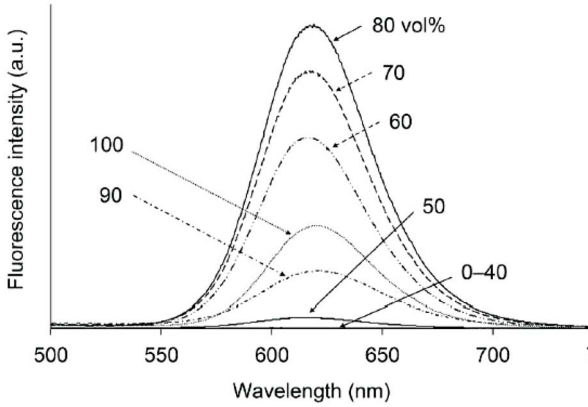


Fig. 2. Fluorescence spectra of **2a** (50 μM) in a mixture of THF and a phosphate buffer at different proportions of the buffer upon excitation at 390 nm.

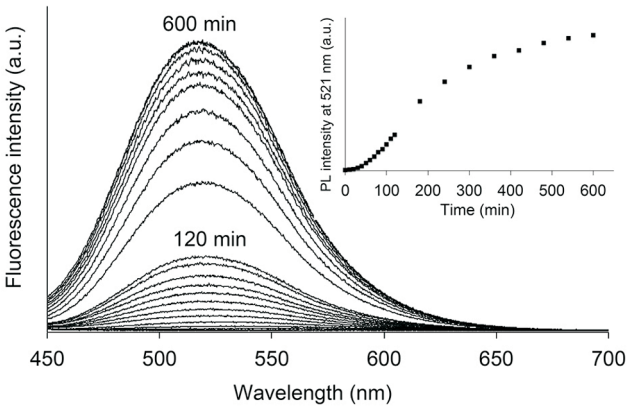


Fig. 3. Time-dependent fluorescence spectra of **2a** (50 μM) in the presence of Cys (3 mM) in phosphate buffer (10 mM, pH 7.2, a mixture with THF, the latter at 60 vol%) upon excitation at 390 nm at 37 °C. Inset: the fluorescence intensity at 521 nm as a function of time.

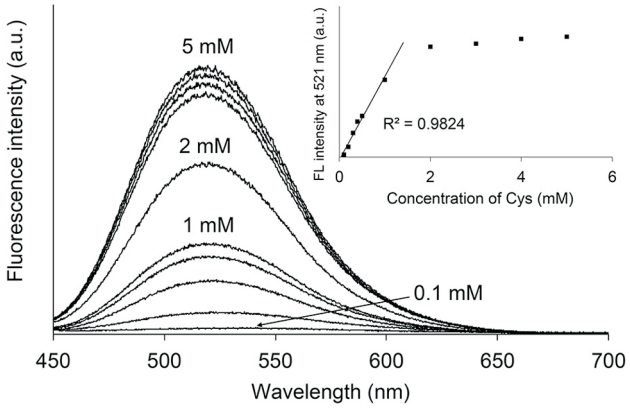


Fig. 4. Fluorescence spectra of **2a** (50 μM) after 2 h upon the addition of Cys at various concentrations (0.1–5 mM) in phosphate buffer (10 mM, pH 7.2, a mixture with THF, the latter at 60 vol%) upon excitation at 390 nm at 37 °C. Inset: fluorescence intensity at 521 nm as a function of Cys concentration.

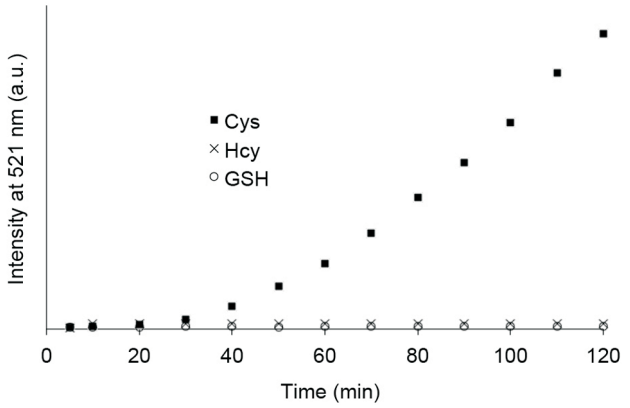


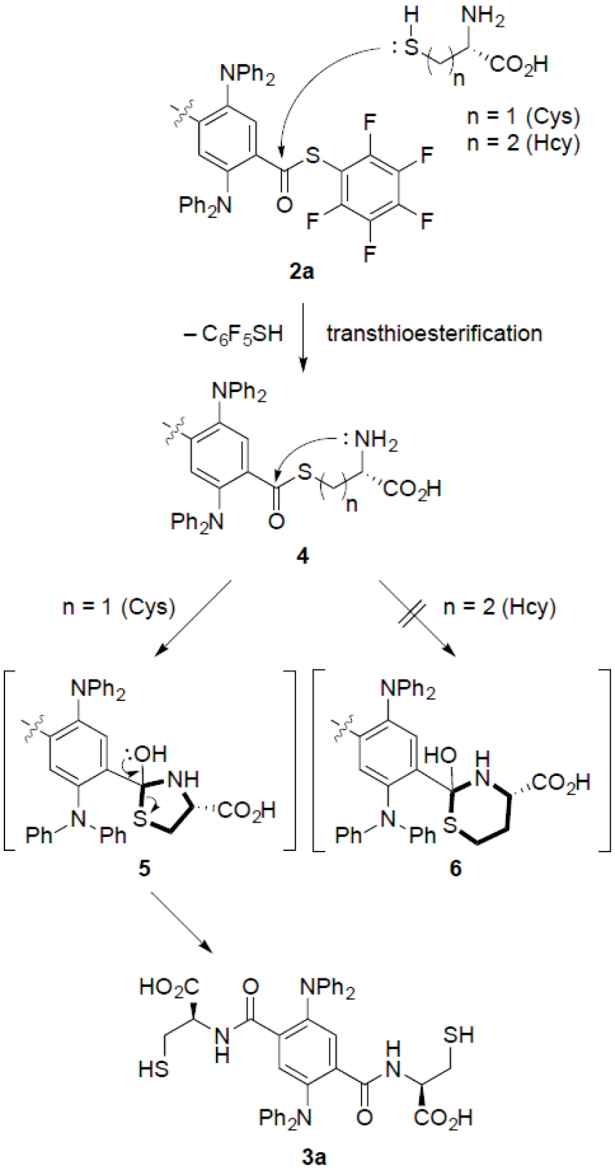
Fig. 5. Fluorescence intensity of **2a** (50 μM) at 521 nm in the presence of Cys, Hcy, or GSH (3 mM) in phosphate buffer (10 mM, pH 7.2, a mixture with THF, the latter at 60 vol%) upon excitation at 390 nm at 37 °C.

resulting mixture was observed even 12 h after the addition of Hcy or GSH (see also Figure S3 in Supporting Information).[9] Thus, **2a** was confirmed to be a Cys-selective fluorescent probe.

The proposed mechanism of the reaction of **2a** with Cys is shown in Scheme 2. First, transthioesterification takes place between **2a** and Cys to generate **4** (n = 1) with releasing C_6F_5SH.[10] After that, the amino group derived from Cys undergoes intramolecular carbonyl addition thus forming thiazolidine intermediate **5**, in which the carbon–sulfur bond can be cleaved by the lone pair of the oxygen attached to the carbon, giving rise to green-fluorescent amide **3a**,[4] whose formation was confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry of the resulting solution (see Figure S5 in Supporting Information). Meanwhile, when Hcy was added to **2a** in THF/PB (60/40), the absorption spectrum of the resulting mixture became different from that of **2a** (see Figure S6 in Supporting Information), indicating that the initial transthioesterification took place successfully as in the case of Cys. The reaction of **2a** with Hcy did not produce green-fluorescent species (Figure 5) probably because formation of tetrahydro-1,3-thiazine **6** is impossible due to the greater steric repulsion between the diphenylamino group and the 6-membered ring at the *ortho*-position.[2i,21]

In summary, we designed and characterized bis(pentafluorophenyl) 1,4-benzenedicarbothionic acid diester as a turn-on fluorescent probe for highly selective sensing of Cys on the basis of NCL. It is remarkable that the probe selectively detects Cys but not Hcy and GSH because NCL-based probes reported so far cannot discriminate Cys and Hcy well. The topics of further research include enhancement of the sensitivity, achievement of a quick response, and application to sensing in live cells.

Scheme 2 Proposed mechanism of Cys detection with **2a**. The left pentafluorophenylthiocarbonyl moieties of **2a**, **3a**, and **4**–**6** are omitted for clarity.



EXPERIMENTAL

General: Melting point was determined using Seiko Instrument Inc. TG/DTA6200. ^{1}H NMR spectrum was measured on a Bruker Avance II 300 (300 MHz) spectrometer. The chemical

shifts of ^{1}H NMR are expressed in parts per million downfield relative to the internal chloroform (δ = 7.26 ppm). Splitting patterns are indicated as s, singlet; d, doublet; m, multiplet. ^{13}C NMR spectrum was measured on Varian Mercury 400 (100 MHz) spectrometer with tetramethylsilane as an internal standard (δ = 0 ppm) or chloroform-*d* (δ = 77.0 ppm). ^{19}F NMR spectrum was measured on a Bruker Avance II 300 (282 MHz) spectrometer with C_6F_6 as an internal standard (δ = –163.7 ppm). Chemical shift values are given in parts per million downfield relative to the internal standards. Infrared spectrum (IR) was recorded on a Shimadzu FTIR-8400 spectrometer. FAB-MS analysis was performed with a JEOL JMS-700 spectrometer. TLC analysis was performed by means of Merck Kieselgel 60 F_{254}. Silica gel column chromatography was carried out using Merck Kieselgel 60 (230–400 mesh). Reagent-grade dichloromethane was passed through two packed columns of neutral alumina and copper oxide under a nitrogen atmosphere before use. Tetrahydrofuran used for UV–vis absorption and fluorescence measurements was purchased from Kanto Chemical Co., Inc. and degassed with argon before use. UV–vis absorption and fluorescence spectra were measured with a Shimadzu UV–2550 spectrometer and Shimadzu RF–5300PC spectrometer, respectively.

Synthesis of 2a: A 100 mL two-necked flask was charged with dimethyl 2,5-bis(diphenylamino)terephthalate (1.98 g, 3.74 mmol), THF (20 mL), EtOH (15 mL), and aq. NaOH (8 M, 15 mL, 120 mmol). The resulting mixture was stirred at 80 °C for 16 h. After the organic solvents were removed in vacuo, aq. HCl was added to the remaining liquid until the pH of the mixture became ca. 1. The precipitates were filtered and dried at 80 °C under reduced pressure, giving rise to 2,5-bis(diphenylamino)terephthalic acid (1.86 g, 3.72 mmol, 99%) as a red solid, which was used in the next step without further purification. A flame-dried 20 mL Schlenk flask was charged with 2,5-bis(diphenylamino)terephthalic acid (0.10 g, 0.21 mmol), CH_2Cl_2 (2 mL), and DMF (three drops). To the solution was slowly added thionyl chloride (0.73 mL, 1.00 mmol) at 0 °C. The resulting mixture was refluxed for 3 h, and then the organic solvents were removed in vacuo. The residue was dried under reduced pressure for 1 h. To the flask were added CH_2Cl_2 (2 mL), pyridine (48 μL, 0.60 mmol), and C_6F_5SH (60 μL, 0.48 mmol). The mixture was stirred at 35 °C for 10 h before quenching with 1 M HCl (10 mL). The aqueous layer was extracted with CH_2Cl_2 (30 mL x 3) and the combined organic layer was dried over anhydrous $MgSO_4$ and removed in vacuo. The crude product was purified by silica gel column chromatography (eluent: hexane/CH_2Cl_2 2:1), giving rise to **2a** (0.16 g, 1.86 mmol, 89%) as a red solid. Mp (dec) 234 °C. R_f 0.73 (hexane/CH_2Cl_2 2:3). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.04–7.10 (m, 12H), 7.29 (dd, J = 8.1, 7.5 Hz, 8H), 7.50 (s, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 123.5, 123.8, 129.5, 130.1, 137.6, 142.2, 146.9, 184.5; ^{19}F NMR ($CDCl_3$, 282 MHz): δ –162.5 – –162.3 (m, 4F), –150.9 (tt, J = 19.7, 2.8 Hz, 2F), –132.0 – –131.9 (m, 4F). IR (KBr): 3433, 3036, 1701, 1589, 1514, 1391, 1279, 1260, 1171, 1094, 982, 856, 756, 694 cm^{-1}. HRMS–FAB (*m/z*): $[M]^+$ calcd for $C_{44}H_{22}O_2N_2F_{10}S_2$ 864.0963; found, 864.0956.

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9. Proline also did not react with **2a** at all.
10. Choice of C_6F_5 as R′ in **2** was essential for the smooth reaction of **2a** with Cys. See Figure S4 in Supporting Information.

